Identification of new urinary gamma-hydroxybutyric acid (GHB) markers applying untargeted metabolomics analysis following placebo-controlled administration to humans

Steuer, Andrea E ; Raeber, Justine ; Steuer, Christian ; Boxler, Martina I ; Dornbierer, Dario A ; Bosch, Oliver G ; Quednow, Boris B ; Seifritz, Erich ; Kraemer, Thomas

Abstract: Gamma-hydroxybutyrate (GHB) is a short-chain fatty acid that occurs naturally in the mammalian brain and that is prescribed as a medication against narcolepsy or used as a drug of abuse. Particularly, its use as a knock-out drug in cases of drug facilitated crimes is of major importance in forensic toxicology. Because of its rapid metabolism and resulting narrow detection windows (<12 h in urine), detection of GHB remains challenging. Thus, there is an urgent call for new markers to improve the reliable detection of GHB use. In the framework of a randomized, placebo-controlled, crossover study in 20 healthy male volunteers, urine samples obtained 4.5 hours post-administration were submitted to untargeted mass spectrometry (MS, QTOF) analysis to identify possible new markers of GHB intake. MS data from four different analytical methods (reversed phase and hydrophilic interaction liquid chromatography; positive and negative electrospray ionization) were filtered for significantly changed features applying uni- and multivariate statistics. From the resulting 42 compounds of interest, eight were finally identified including conjugates of GHB with carnitine, glutamate, and glycine as well as the endogenous compounds glycolate and succinylcarnitine. While GHB conjugates were only detectable in the GHB, but not in the placebo group, glycolate and succinylcarnitine were present in both groups albeit significantly increased through GHB intake. Untargeted metabolomics proved as a suitable tool for the non-hypothesis driven identification of new GHB markers. However, more studies on actual concentrations, detection windows, and stability will be necessary to assess the suitability of these markers for routine application.

DOI: https://doi.org/10.1002/dta.2558

Identification of new urinary gamma-hydroxybutyric acid (GHB) markers applying untargeted metabolomics analysis following placebo-controlled administration to humans

Andrea E. Steuer[1], Justine Raeber[1], Christian Steuer[2], Martina I. Boxler[1], Dario A. Dornbierer[1, 3, 4,5], Oliver G. Bosch[4], Boris B. Quednow[4], Erich Seifritz[4, 5], Thomas Kraemer[1]

[1] *Department of Forensic Pharmacology & Toxicology, Zurich Institute of Forensic Medicine, University of Zurich, Switzerland*

[2] *Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH), Switzerland*

[3] *Institute of Pharmacology and Toxicology, University of Zürich*

[4] *Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zürich*

[5] *Zürich Center for interdisciplinary Sleep Research (ZiS), University of Zürich*

Corresponding author:

PD Dr. Andrea E. Steuer

University of Zurich

Zurich Institute of Forensic Medicine (ZIFM)

Department of Forensic Pharmacology & Toxicology

Winterthurerstrasse 190/52

CH-8057 Zurich

Switzerland

Tel.: 0041 446355679; fax: 0041 446356852

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/dta.2558

E-mail address: andrea.steuer@irm.uzh.ch

OrcID 0000-0001-8983-2353

Abstract

Gamma-hydroxybutyrate (GHB) is a short-chain fatty acid that occurs naturally in the mammalian brain and that is prescribed as a medication against narcolepsy or used as a drug of abuse. Particularly, its use as a knock-out drug in cases of drug facilitated crimes is of major importance in forensic toxicology. Because of its rapid metabolism and resulting narrow detection windows (<12 h in urine), detection of GHB remains challenging. Thus, there is an urgent call for new markers to improve the reliable detection of GHB use. In the framework of a randomized, placebo-controlled, crossover study in 20 healthy male volunteers, urine samples obtained 4.5 hours post-administration were submitted to untargeted mass spectrometry (MS, QTOF) analysis to identify possible new markers of GHB intake. MS data from four different analytical methods (reversed phase and hydrophilic interaction liquid chromatography; positive and negative electrospray ionization) were filtered for significantly changed features applying uni- and multivariate statistics. From the resulting 42 compounds of interest, eight were finally identified including conjugates of GHB with carnitine, glutamate, and glycine as well as the endogenous compounds glycolate and succinylcarnitine. While GHB conjugates were only detectable in the GHB, but not in the placebo group, glycolate and succinylcarnitine were present in both groups albeit significantly increased through GHB intake. Untargeted metabolomics proved as a suitable tool for the non-hypothesis driven identification of new GHB markers. However, more studies on actual concentrations, detection windows, and stability will be necessary to assess the suitability of these markers for routine application.

Keywords: GHB, carnitine, untargeted metabolomics, placebo-controlled

Introduction

Gamma-hydroxybutyrate (GHB) is a short-chain fatty acid that occurs naturally [1, 2], but is also exogenous applied as a drug of abuse (DOA) or as a prescription medication. Clinically, the drug is internationally approved for the treatment of narcolepsy with cataplexy, as it improves sleep impairments and daytime sleepiness in these patients, and significantly reduces cataplectic attacks[3]. GHB and its precursors γ-butyrolactone (GBL) and 1,4-butanediol are also consumed/abused recreationally as DOAs. In forensic toxicology, particularly their use as so-called *date rape* or *knock-out* drug in cases of drug facilitated crimes (DFC) or drug facilitated sexual assaults (DFSA) is of importance[4, 5].

In vivo, GHB is rapidly converted to succinic semialdehyde followed by subsequent oxidation to succinate, an intermediate of the Krebs cycle[6]. Further, GHB is catabolized by processes that include direct β-oxidation resulting in acetyl-CoA and glycolate and α-oxidation forming 3-hydroxypropionyl-CoA[7, 8]. Recently, formation of phase II metabolites of GHB, namely GHB-glucuronide and GHB-sulfate have been described[9, 10]. An overview of the main pathways involved in GHB catabolism/metabolism is depicted in Fig. 1. Due to its rapid metabolism and therefrom resulting narrow detection windows of only up to 6 hours in plasma and less than 12 hours in urine[11, 12], detection of GHB, particularly in DFSA cases, remains challenging. Particularly discrimination between endogenous and exogenous GHB is complicated, especially in those samples with low concentrations. Numerous authors recommend cut-offs to facilitate such a decision. However, a standard procedure has not yet been specified[4].

Current research interest focuses on new metabolites or new methodologies to prolong the detection window after GHB intake/administration and to improve the differentiation between endogenous levels and exogenous administration. The phase II metabolites GHB-glucuronide and GHB-sulfate have been evaluated for that purpose, assuming phase II conjugates to have longer half-lives than the parent drug[9, 10]. While both metabolites could be detected, comprehensive pharmacokinetic data on their elimination after controlled GHB administration are still missing. Petersen et al. found large inter-individual differences in reference ranges of endogenous GHB-glucuronide concentrations in

urine, but did not measure any authentic GHB positive cases[10]. Mehling et al. could demonstrate increased GHB-glucuronide levels in urine samples of three subjects, however, the concentrations were generally not higher than endogenous concentrations measured in a control group[13]. Hanisch et al. suggested the existence of a sulfonated GHB metabolite, GHB-sulfate, and were able to detect it in authentic urine samples[9]. But again, further studies on GHB-sulfate also indicated that the large inter-individual variability of concentrations found in a reference population makes GHB-sulfate itself unsuitable to prolong the detection times of GHB applications[14]. So far, neither GHB-glucuronide nor GHB-sulfate proved suitable to ultimately confirm exogenous GHB consumption or prolong its detection window[13, 14]. A completely new and interesting strategy has been recently described by Mehling et al., trying to assess differential gene expressions after GHB intake to identify potential biomarkers for the detection of GHB intake. However, because of the low statistical power of the small sample size, alterations in gene expression relating to GHB intake could not be confirmed to a forensically sufficient degree[15]. Thus, the identification of novel biomarkers of GHB consumption remains of highest interest.

Metabolomics research focuses on high-throughput identification of a multitude of small molecular weight molecules in one single sample. Mapping the biochemical changes after DOA exposure may complement traditional approaches by revealing potential biomarkers of organ toxicity, discovering new metabolites in a time- and dose-dependent manner and different pharmacodynamic targets. This approach can also help by giving insights about the pathways implicated in the mechanism of drug action, adverse effects and variability of the drug response[16]. A few metabolomic studies have been performed in order to further evaluate the underlying mechanisms of GHB's pharmacological action[17, 18] or to finally find useful biomarkers of its consumption[14, 18, 19]. For example, Luca et al. found up to sevenfold higher levels of glycolate – originating form β-oxidation of GHB - in brain and liver of mice[18]. Also Palomino-Schätzlein et al. observed significant increases in glycolate as well as succinate in urine following controlled administration of GHB to humans using nuclear magnetic resonance (NMR)-based metabolomics[19]. Piper et al. applied mass spectrometry (MS)-based metabolomics while searching for endogenous urinary metabolites potentially correlating with the

urinary excretion of GHB-sulfate and GHB-glucuronide in order to find a suitable marker to normalize urinary concentrations[14].

Although initial attempts have recently been made to find potential biomarkers of GHB, forensic toxicology is still in need for a reliable marker to confirm the consumption of GHB and its substitutes, particularly over longer time frames. Therefore, the exclusive aim of the current study was to identify possible new markers of GHB intake by evaluating urine samples obtained in the framework of a randomized, placebo-controlled, crossover study of GHB administration to 20 healthy male volunteers who took the study medication at night in the middle of a sleep episode. Urine was subsequently sampled after awakening in the morning. While it is obvious that the GHB study design was not suitable to find a marker that can prolong the time of detection, the relatively short time between application of GHB and the urine sampling (4.5 h) ensured the presence of the parent compound and the possibility to find any new markers for GHB intake. Thus, long time detectability was not part of the present study. The new markers should be identified as part of a comprehensive MS-based, untargeted metabolomics approach. The further investigation of other metabolomic changes after GHB intake was not part of the current study.

Materials and Methods

Participants

The study followed a randomized, balanced, double-blinded placebo-controlled crossover design as described in detail elsewhere (Dornbierer DA, Boxler M, Voegel CD, Stucky B, Steuer AE, Binz TM, Baumgartner MR, Baur DM, Quednow BB, Kraemer T, Seifritz E, Landolt HP, Bosch OG. Noctural gamma-hydroxybutyrate reduces cortisol awakening response and morning tryptophan catabolites (TRYCATs) in healthy volunteers, under review) and was approved by the Cantonal Ethics Committee of Zurich as well as the Swissmedic. The study was registered at ClinicalTrials.gov (NCT02342366). All participants were instructed about potential risks concerning the administered substance and provided written informed consent. They received a monetary compensation for the completion of the study.

Two experimental nights per session were separated by a washout phase of seven days. Each session consisted out of three nights: an adaptation night, an experimental night – where patients either received 50 mg/kg bodyweight of sodium oxybate (Xyrem®) or placebo – and a follow-up night. Twenty healthy, male volunteers (mean age 25.8±2.45 years) participated in the study. Following criteria were required for inclusion: (i.) male sex to avoid the potential impact of menstrual cycle on blood chemical variables, (ii.) age within the range of 18 to 30 years, (iii.) absence of any somatic or psychiatric disorders, (iv.) no first-degree relatives with a history of psychiatric disorders, (v.) non-smoking, (vi.) without a history of drug abuse (lifetime use >5 occasions, with exception of occasional cannabis use). None of the participants reported previous experiences with GHB in their life. Participants had to restrain from illegal drugs for two weeks and from caffeine for one week prior to the first and until the second experimental night. No alcohol was allowed 24 h before each experimental night.

Procedure

GHB was administered at the beginning of the second half (2:30 am) of the experimental night. Therefore, subjects were woken up at 2:30 am and each subject received 50 mg/kg of GHB (Xyrem®) dissolved in 2 dl of orange juice anda placebo (each participant underwent two session, one with placebo, one with GHB, see section Participants), matched in appearance and taste. After that, subjects were allowed to return to sleep. The used dose represents the maximal starting dose used for the treatment of narcolepsy (compendium.ch). Early morning urine was collected after the adaptation night and the experimental night (7:00 am) and stored at -80 °C till analysis (maximum of 24 months).

Chemicals and Reagents

GHB was obtained from Lipomed (Arlesheim, Switzerland) and GHB-glucuronide from Reseachem (Burgdorf, Switzerland). 1-Methylhistidine, adenine, adenosine, arginine, azelaic acid, butyrylcarnitine, carnitine, chenodeoxycholic acid, cholic acid, citrulline, cortisol, cortisone, creatinine, deoxycholic acid, glutaric acid, glycolic acid, glycocholic acid, hippuric acid, inosine, isoleucine, leucine, L-pyroglutamic acid, methionine, methylmalonic acid, mevalonolactone, N,N-

dimethylglycine, nicotinic acid, p-aminobenzoic acid, phenylalanine, proline, raffinose, riboflavin, taurine, taurocholic acid, tryptophan, and uracil were purchased from Sigma-Aldrich (Buchs, Switzerland). Deuterated and heavy-labeled internal standards (IS) adenosine ribose-D_1, arginine-$^{13}C_6$, caffeine 3-methyl-^{13}C, carnitine trimethyl-D_9, creatinine N-methyl-D_3, deoxycholic acid-D_4, D-fructose ^{13}C, glycine-$^{13}C_2$, glycocholic acid-D_4, hippuric acid ^{15}N, kynurenine-D_4, leucine-D_{10}, lysine-D_4, phenylalanine-D_1, proline ^{15}N, serine-D_3, tryptophan-D_5 and uric acid-$^{15}N_2$ were purchased from Cambridge isotope laboratories, which were delivered by ReseaChem Life Science (Burgdorf, Switzerland) or Sigma-Aldrich (Buchs, Switzerland). Water, acetonitrile (ACN), methanol (MeOH) of HPLC grade were obtained from Fluka (Buchs, Switzerland). All other chemicals used were from Merck (Zug, Switzerland) and of the highest grade available.

Sample preparation

Urine samples of the experimental nights after placebo and GHB intake of 19 participants (sample of one participant was unavailable) were thawed at room temperature and vortexed for 20 seconds. Forty µL of the IS mix (adenosine ribose-D_1 15 µmol/L, arginine-$^{13}C_6$ 300 µmol/L, caffeine 3-methyl-^{13}C 200 µmol/L, carnitine trimethyl-D_9 100 µmol/L, creatinine N-methyl-D_3 500 µmol/L, deoxycholic acid-D_4 1.8 µmol/L, D-fructose ^{13}C 120 µmol/L, glycine-$^{13}C_2$ 800 µmol/L, glycocholic acid-D_4 150 µmol/L, hippuric acid ^{15}N 500 µmol/L, kynurenine-D_4 8 µmol/L, leucine-D_{10} 300 µmol/L, lysine-D_4 700 µmol/L, phenylalanine-D_1 300 µmol/L, proline ^{15}N 700 µmol/L, serine-D_3 450 µmol/L, tryptophan-D_5 250 µmol/L and uric acid-$^{15}N_2$ 500 µmol/L)[20] was placed into autosampler filter vials (0.45 µm PTFE, Thomson Instrument company, California, USA) and 200 µL of urine sample were added. Depending on the acquisition method either 200 µL of a 1:1 (v/v) mixture of eluents A and B (for measurement on the HSST column) or C and D (for measurement on the HILIC column,see below) was added. After mixing, the filter vials were carefully closed with a filter plunger and were either analyzed directly as described below or stored at -20 °C until analysis. A mix of all urine samples (200 µl each) of the clinical study was prepared (pooled sample) and prepared as described above.

UHPLC-HRMS

In total 38 samples (19 participants, 2 sessions) were subjected to MS measurements in randomized order on a Thermo Fischer Ultimate 3000 UHPLC system (Thermo Fischer Scientific, San Jose, CA) coupled to a high-resolution (HR) TOF instrument system (TripleTOF 6600, Sciex, Concord, Ontario, Canada). Mobile phases A and B consisted of 10 mM ammonium formate with 0.1 % (v/v) formic acid in water and 0.1 % (v/v) formic acid in MeOH, respectively. Mobile phases C and D were 25 mM ammonium acetate and 0.1 % (v/v) acetic acid in water and 0.1 % (v/v) acetic acid in ACN, respectively.

Two different columns – reversed phase and hydrophilic interaction liquid chromatography (HILIC) were used for chromatographic separation. A Waters (Baden-Daettwil, Switzerland) XSelect HSST RP-C18 column (150 mm x 2.1 mm, 2.5 µm particle size) was applied with the following gradient: 1 min 100% A; 1-15 min 100% B; 15-18 min 100% and then decreased to start conditions and re-equilibration for 2 min. Flow rate increased after 15 min to 0.7 ml/min. Further a Merck SeQuant ZIC HILIC column (150 mm x 2.1 mm, 3.5 µm particle size) using the following gradient: 1 min 95% D; 1-10 min decrease to 40% D; 10-12 min decrease to 10% D; 12-13 min 10% D; and then increase to start conditions and re-equilibration for 2 min was used. The column oven was set to 40 °C and injection volume was 1 µl for all samples.

High resolution mass spectra (MS) and MS/MS data were acquired by two methods: TOF MS only and information dependent data acquisition (IDA) in positive and negative ionization mode. MS analysis was performed with a DuoSpray ion source at a resolving power (full width at half-maximum at m/z 400) of 30'000 in MS and 30'000 in MS2 (high-resolution mode) or 15'000 (high-sensitivity mode) in positive ionization mode. Automatic calibration was obtained every fifth sample injections using atmospheric-pressure chemical ionization (APCI) positive calibration solution (Sciex) in the positive ionization mode and every three sample injections using APCI negative calibration solution (Sciex) in the negative ionization mode. The TOF MS method was composed of a TOF-MS scan over a mass range from m/z 50 to m/z 1000 (accumulation time 100 ms, collision energy (CE) 5 eV). Additionally, about 20% of the samples were measured in the IDA scan mode. The IDA method consisted of a TOF-MS scan over a mass range from m/z 50 to m/z 1000 (accumulation time 50 ms,

CE 5 eV). IDA experiments (accumulation time for each IDA experiment 100 ms, CE 35 eV with a CE spread of 15 eV) were performed after dynamic background subtraction on the four most intense ions with an intensity threshold above 100 counts per second (cps) and exclusion time of 5 s (half peak width) after two occurrences in high sensitivity mode.

A system suitability test (SST) containing 1-methylhistidine, adenine, adenosine, arginine, azelaic acid, chenodeoxycholic acid, cholic acid, citrulline, cortisol, cortisone, creatinine, deoxycholic acid, glutaric acid, glycocholic acid, hippuric acid, inosine, isoleucine, leucine, L-pyroglutamic acid, methionine, methylmalonic acid, mevalonolactone, N,N-dimethylglycine, nicotinic acid, p-aminobenzoic acid, phenylalanine, proline, raffinose, riboflavin, taurine, taurocholic acid, tryptophan and uracil (concentration 10 µg/ml each) was measured after every fifth sample. The SST was checked for reproducibility of the data by retention time (RT) shifts and peak area comparison using MultiQuant V 2.1 (Sciex). Further, a pooled sample was additionally measured after every fifth sample.

Targeted data evaluation for GHB, GHB-glucuronide, and GHB-sulfate

Targeted analysis from the untargeted data set on GHB and its phase II metabolites GHB-glucuronide and GHB-sulfate was performed by obtaining peak areas using MultiQuant 2.1 (Sciex). Analyte peak areas were normalized to creatinine (determined by the Jaffe reaction on an Indiko Plus device, Thermo Scientific, Braunschweig, Germany) of each urine sample. Statistical comparison by paired t-tests ($p < 0.05$) was performed in GraphPad Prism 7 (GraphPad Software, CA, USA).

Feature selection/statistical evaluation

Progenesis QI (Waters Corp, Milford, USA) was used for data-preprocessing, alignment, deconvolution, peak picking, initial data normalization and filtering on TOF data only. Data files of the IDA scan mode were incorporated in the software for identification purposes only. Via automatic processing, a reference run was picked based on its similarity to all the other runs and used as a reference for retention time alignment. Peak picking parameters were as follows: automatic sensitivity method, sensitivity value 3, no minimum peak width and no retention time limits. Features were

automatically deconvoluted based on the same retention time and ion masses that differ by an amount equal to the mass difference between 2 experiment adducts. All samples were normalized to the feature identified as creatinine in each method. All four data sets (HSST ESI^+, HSST ESI^-, HILIC ESI^+, HILIC ESI^-) were initially filtered for features with MS/MS information from respective IDA files available and an abundance > 1000 cps in positive and > 500 cps in negative mode, respectively. Paired t-test ($p < 0.05$) and partial least square discriminant analysis (PLS-DA) was applied on log-transformed and auto-scaled data after initial data filtering by median intensity values in MetaboAnalyst 4.0[21].

Compound identification

Significant features were searched on the MS and MS/MS level against an in-house database, national institute of standards and technologies (NIST) and Chemspider in Peakview 2.2 as well as in the online databases METLIN (https://metlin.scripps.edu) and the human metabolome database[22] in Progenesis QI. Confirmation of identity was performed by library search matching (library fit search score, > 90%) of the accurate mass of precursor and fragment ions and retention time values to authentic standards if available.

Final identification results were classified on the different levels of identification confidence suggested by the metabolomics standard initiative (MSI)[23]: level 1 provides identified compounds and uses two or more measured orthogonal parameters (e.g. retention time and mass spectrum) of an authentic chemical standard analyzed under the identical analytical conditions that matches the metabolite present in the sample. Level 2 provides putatively characterized compounds and identification is based on physicochemical properties and/or similarities with mass spectra of public or commercial libraries. Level 3 provides putatively characterized compound classes by spectral similarities to known compounds of a chemical class or based upon physicochemical properties of a chemical class.

Authentic GHB urine samples

Thirty routine urine samples (n=10 GHB positive, n=20 control samples) send to the author's lab by police or state attorneys were reanalyzed anonymized in full conformance with Swiss laws (statement of Cantonal Ethics Board of the Canton of Zurich: BASEC-Nr. Req-2017-00946). GHB had been tested during case work in all samples using a GHB enzymatic assay (cut-off 40 mg/L, in-house validated) from Bühlmann (Schönenbuch, Switzerland) on an Indiko Plus device (Thermo Scientific, Braunschweig, Germany). Positive findings had been confirmed by a routine gas chromatography (GC)-MS analysis (liquid-liquid extraction with ethylacetate; derivatization with N-Methyl-N-(trimethylsilyl) trifluoroacetamide; GHB-d6 as IS). Creatinine was determined by the Jaffe reaction on an Indiko Plus device. Samples were initially assigned as GHB positive if the immunoassay result was > 40 mg/L and presence of GHB was confirmed by GC-MS (measured urine concentration range: 76 – 12000 mg/L). Targeted analysis on previously identified markers was performed in MultiQuant 2.1 (Sciex).

Results and Discussion

Although initial attempts have recently been made to find potential biomarkers for GHB, forensic toxicology is still in need for a reliable marker other than GHB itself to confirm the consumption of GHB particularly over longer time-frames. MS-based, untargeted metabolome analysis is an interesting emerging analytical technique for biomarker research. The current study strictly focuses on the identification of potential new biomarkers for GHB showing large differences between placebo and GHB groups from a placebo-controlled crossover study.

Analytical procedure

A universal LC-HRMS method was selected that is typically applied in metabolomics studies[24, 25]. Two different chromatographic systems – reversed phase (HSST) and HILIC – in both positive and negative ionization mode were chosen to cover as many small molecules as possible. The applied screening method has been extensively examined recently on analyte selection, detectability and

sensitivity [20] and was already successfully applied to other untargeted and targeted studies of the metabolome[26-28]. For peak picking and deconvolution, measurements were only performed in TOF-MS mode, while additional runs were performed after IDA MS/MS acquisition for identification. Analytical performance was monitored over each whole batch through regular analysis of SST and pooled samples. Mean coefficient of variation (CV) of the peak area of all SST compounds for the four methods were 7.5 +/- 1.5 for HSST ESI^+, 14 +/- 4.3 for HSST ESI^-, 33+/- 18 for HILIC ESI^+, and 16 +/- 8.8 for HILIC ESI^-, respectively and were overall comparable to former studies[28]. Pooled samples' CVs were higher when calculated for all detected features. However, the large majority of all features were of very low abundance. In the current study, only features with an abundance of at least 1000 cps in positive and 500 cps in negative mode were further evaluated. For these features, variation was < 30% for more than 80% of all features. Overall, all experiments were reproducible in terms of peak intensity and retention time continuity and were therefore used for the analysis of the data. Additionally, all samples were analyzed in random order to avoid systematic effects resulting from varying instrument performance during runtime of the batch.

Targeted analysis of GHB, GHB-glucuronide and GHB-sulfate

Prior to untargeted data evaluation, targeted analysis on GHB and its previously described metabolites GHB-glucuronide and GHB-sulfate was performed from the untargeted data acquisition. It has to be mentioned that the applied analytical methods had not been optimized for GHB or its metabolites. Nevertheless, GHB as well as its glucuronide and sulfate conjugate could be detected in all urine samples of the GHB group using HILIC chromatography and negative ionization. Hanisch et al were unable to reproducibly measure GHB-sulfate in negative mode[9] which was not observed in the present work. The chromatography for the three analytes is given in the supplementary information Fig. S1. Although GHB is only shortly retained, it was sufficiently separated from two isomers (presumably α- and β-hydroxybutyric acid). Identity of GHB and GHB-glucuronide was confirmed based on accurate mass, MS/MS and retention time comparison to reference material. GHB-sulfate has not been commercially available, but could be nevertheless identified by its accurate mass and interpretation of its MS/MS spectrum. The MS/MS spectra are given in Fig. S2. No actual quantification was

performed, as samples were only analyzed in the untargeted metabolome method. Therefore, all results are given on analyte peak area over creatinine ratios. Results for the comparison between placebo and GHB administration are given in Fig. 2 for the group as a whole and additionally for each single subject in the supplementary information Fig. S3. As expected, GHB significantly increased after GHB administration. On the contrary, no significant differences between treatments could be observed for GHB-glucuronide and GHB-sulfate. Systematic studies on the pharmacokinetics of GHB phase II metabolites under controlled conditions are scarce and limited to small sample sizes (n=3) or authentic (and thus not controlled) samples only. While increases in GHB-glucuronide and GHB-sulfate were sometimes observed[13, 14], the concentrations were generally not different from endogenous levels. Tittarelli et al. also could not observe significant differences of GHB-glucuronide concentrations in plasma to control patients[29].

In the present study, individual subjects also showed trends for higher GHB-glucuronide and/or higher GHB-sulfate levels, but overall the effect was negligible. We further confirmed our findings through additional analysis of authentic samples, which confirmed the study results regarding GHB-glucuronide and GHB-sulfate (Fig. S4). The reason for GHB-glucuronide and GHB-sulfate being unaffected by treatments remains unclear. It appears possible, that the responsible enzymes, UDP-glucuronyltransferases and sulfotransferases, might be already saturated by endogenous GHB concentrations, although no data on involved isoenzymes, respective affinities (K_m) and enzyme capacities (V_{max}) are available. As already proposed by others, our data propose that GHB-glucuronide and GHB-sulfate are not suitable to monitor GHB consumption[13, 14].

Untargeted metabolomics analysis: selection of potential interesting features

Prior to feature filtering and selection, normalization to creatinine was applied as typically used for urine specimens. Typical metabolomics analysis results in large amounts of data. To improve data handling, initial analysis focused only on features with MS/MS data from IDA experiments available, resulting in 194, 111, 55 and 101 picked features for HSST ESI^+, HSST ESI^-, HILIC ESI^+ and HILIC ESI^-, respectively.

Further advanced statistical evaluation of the data was performed with MetaboAnalyst[30]. Data reduction by median should further remove features close to baseline levels which will be unsuitable as reliable biomarker. The data were further log transformed and auto-scaling (mean-centering and division by the standard deviation of each variable) was applied. With this, uniform distribution of the data was achieved, as also described previously[27, 28]

Features showing significant differences in paired t-test analysis were initially considered of particular interest for further identification. Additionally, features showing the highest variable importance parameters (VIPs) scores per method (first 15 each) determined in PLS-DA analysis were additionally considered. The higher the VIP score, the higher the contribution of a certain feature to separate placebo from GHB intake. Those features with analyte peak area/creatinine peak area intensity < 1000 cps ESI^+; < 500 cps ESI^- were omitted, as they had large CVs and were unlikely to provide reproducible results.

Some features were detected under more than one condition, e.g., compound 8, that was detected both in HSST ESI^+ and HILIC ESI^+ or compound 14 that was detected in HSST ESI^+ and ESI^-. These features were combined to one single compound. In the end of the feature selection process, 42 compounds fulfilled the described criteria and are given in Table 1, arranged according to their median fold-changes between placebo and GHB group. GHB itself (compound 7) was identified applying the described feature selection workflow proving its general applicability to pick features of interest. As already expected from the targeted data analysis, neither GHB-glucuronide nor GHB-sulfate made it to that list due to missing significant differences between placebo and GHB group.

Identification

Out of the 42 total compounds that were subjected to MS/MS identification, eight could be tentatively identified and are presented in Table 1. The corresponding MS/MS spectra of four selected compounds (compound 1, 5, 8, and 38) are depicted in Fig. 3. Identification was based on accurate precursor mass ($M+H^+$ / $M-H^-$) and interpretation of accurate fragment ions calculating their molecular composition and respective ppm deviation. Identification levels were given according to the metabolomics standard initiative (MSI)[23] in Table 1. For glycolate (compound 19) identity was

unambiguously confirmed by comparison of retention time and mass spectral information to a commercially available reference standard. Compound 1 and 5 were identified as amino acids conjugates of GHB, namely with glycine (compound 5) and glutamate (compound 1), while compound 8 represents the carnitine conjugate of GHB.

Next to the eight (tentatively) identified compounds, no clear identifications were possible. One point that should be kept in mind is the presence of the adjuvant malic acid in the administered GHB preparation. Of course, malic acid itself or metabolites/conjugates of malic acid might massively differ between placebo and GHB group. So far, neither malic acid itself nor straight forward conjugations products could be identified.

Newly identified GHB markers

Different types of markers were identified as given exemplarily in Fig. 4. Additional information on changes of each single subject is given in Fig. S5. Identified compounds of particular interest will be discussed separately in the following subchapters. In general, some markers appear to be not present in control samples, but are formed in the presence of exogenous GHB, such as GHB-carnitine (compound 8), GHB amino acid conjugates (compounds 1 and 5), and several still unknown compounds (e.g., neutral mass 340.12). Such compounds are more likely direct derivatives or even metabolites of GHB and present the most promising candidates as markers for routine applications. As already discussed above, also derivatives of the adjuvant malic acid might appear only in the GHB group, but would be of no value in real cases, where GHB containing products other than Xyrem® are consumed. Therefore, only markers unambiguously identified are currently of interest. Several other compounds are present both in the control group and after GHB treatment albeit in different amounts. Typically, increased concentrations were observed, e.g., for glycolate (compound 19) and succinylcarnitine (compound 38), while only compound 42 showed concentration decreases.

GHB-Carnitine

Compound 8 was identified as GHB-carnitine, the ester between GHB and L-carnitine. L-Carnitine (beta-hydroxy-gamma-trimethylaminobutyrate) is a small and highly polar zwitterionic molecule that plays a critical physiological role in β-oxidation and energy metabolism by translocating long chain fatty acids across the mitochondrial inner membrane[31-33]. Carnitine conjugation as a metabolic reaction for xenobiotics is rarely described[34] and was initially shown for valproic acid [35]. Considering the chemical structures of GHB and carnitine, theoretically formation of two isomers is possible: esterification between the hydroxy group and/or the carboxyl group of carnitine. Millington et al discussed *in vivo* formation of valproic acid carnitine to be catalyzed through the carnitine acyltransferase on the activated intermediate valproyl-CoA[35]. The structural similarity of GHB to butyric acid and endogenous occurring α- and β-hydroxy butyric acids makes its conjugation to carnitine via acyltransferase very likely. With an underlying enzymatic process, esterification between the carboxylic function of GHB with the beta-hydroxy group of carnitine in analogy to butyrylcarnitine or β-hydroxy butyrylcarnitine seems highly likely. This assumption is supported by the underlying mass spectra depicted in the Fig.S6. Comparable fragmentation pattern of the newly identified GHB-carnitine to β-hydroxy butyrylcarnitine and butyrylcarnitine were observed. The main fragment of *m/z* 85 results from a McLafferty rearrangement of the butyric acid side chain (carnitine) to *m/z* 144 followed by the loss of the trimethylamine moiety (-59)[36]. The *m/z* 103 results from the remaining GHB moiety. A major difference in abundance could be observed in the neutral loss of the carnitine backbone (-161) resulting in an *m/z* of 87 for GHB-carnitine and *m/z* 71 for butyrylcarnitine. In case of GHB-carnitine the resulting carbonyl-fragment can be stabilized by hydrogen bonds explaining its higher abundance.

In contrast to GHB-glucuronide and GHB-sulfate, there seems to be no saturation effects of the carnitine acyltransferase as intake of GHB triggered high formation of this metabolite. However, currently little is known on the enzyme capacity towards GHB. Also non-enzymatic formation of GHB-carnitine seems possible. Considering its peak area, GHB carnitine appears as the most promising marker for GHB intake from the current data set. Of course, data on actual concentrations and particularly of detection windows are still missing and are desperately needed. In the present

study, only urine samples collected 4. 5 hours post GHB administration have been available, a time frame in which GHB itself can still be detected.

Another advantage of GHB carnitine might be its ability to be detectable in positive ionization mode. Routine toxicological screening by LC-MS is often only performed following positive ionization and usually unable to detect GHB reliably. Integration of GHB-carnitine into routine LC-MS screening methods could allow for simultaneous detection of GHB consumption/administration in the very same run with common drug screening.

GHB-Amino acid conjugates: GHB glutamate and GHB glycine

Compound 1 and 5 were identified as level 3 identifications using HILIC chromatography and negative ionization as amino acid conjugates. Conjugation of xenobiotic carboxylic acids with endogenous amino acids has been shown to be an important metabolic pathway in the biotransformation of a number of compounds in a variety of species. The reaction involves the formation of an amide or peptide bond between the carboxyl group of the xenobiotic acid and the amino group of the endogenous compound. The conjugation reaction is generally accepted to be a two-step process starting with the same initial activation to acyl-CoA thioester as described for carnitine conjugation; followed by an acyl transfer to the amino group of an amino acid[37]. Since the initial observation of glycine conjugation to yield hippuric acid from benzoic acid, a number of alternative amino acids have been shown to be involved in amino acid conjugation. The amino acid utilized for conjugation is highly dependent on both the structure of the xenobiotic carboxylic acid and the species under investigation. The most frequently observed amino acid conjugates are those with glycine[37-39]. Compared to the detected GHB-carnitine, the abundance of GHB-glycine and particularly GHB-glutamate was much lower and might even be too low for routine applicability.

Glycolate and succinylcarnitine

While present in both treatment groups, glycolate and succinylcarnitine significantly increased in concentration due to GHB administration. Palomino-Schätzlein et al recently also observed significant concentration increases of glycolate and succinate in urine samples after controlled GHB

administration[19]. While succinate itself was not identified in the current study, an increase in succinylcarnitine, the carnitine conjugate of succinate was observed as a follow-up metabolite of succinate. Looking at the (endogenous) metabolism of GHB (Fig.1), elevated concentrations of compounds within these pathways seem reasonable after GHB intake. However, although significant differences (in paired samples) could be observed, it appears unlikely that under physiological conditions, taking inter-individual variations into account, an increase in glycolate, succinate or succinylcarnitine will not be sufficient to prove GHB intake.

Identified marker in authentic routine samples

A brief proof of concept experiment was performed monitoring the newly identified GHB markers in 30 authentic samples (20 negative, 10 positive for GHB). Although the sample size was too small for reliable sensitivity and specificity calculation, a first proof of suitability of the markers was possible within a detection window where GHB itself is still detectable. As exemplified in Fig. 5 for GHB-carnitine, it was undetectable in control samples, but present in 9 of 10 GHB positive specimens. Similarly, GHB-glutamate (compound 1) and GHB-glycine (compound 5) were only detected in the 10 GHB positive specimens but in none of the controls. There was no correlation between urinary GHB concentration and presence or absence of the new GHB adducts. As the GHB samples were analyzed after anonymization – no further information of GHB dose or time since last intake was available. The selection bias in all samples was of course the analytically confirmed presence of GHB itself, pointing to recent GHB ingestion.

Limitations of the study

Our study had some limitations. Only men were allowed as study participants, while victims of DFSA cases typically are women. Urine samples were collected 4.5 hours post-administration, thus the potential of the identified markers to prolong GHB's detection window cannot be concluded at date. So far, GHB-adducts with carnitine and amino acids could be only detected in a small number of cases were GHB itself was still present. In the controlled study design all participants received GHB in form of Xyrem®, while on the illegal market often GBL is consumed / administered. Considering

its rapid metabolism to GHB in the body, similar formation of adducts is expected nevertheless. In general, the applied GHB dose was rather low compared to doses administered in DFSA cases. Finally, the focus of the study was only on features with MS/MS data available from the screening approach, which might have excluded identification of other interesting markers simply not triggered by the applied MS methods.

Conclusion

Using untargeted metabolomics strategies, three new and promising GHB-metabolites, namely GHB-carnitine, GHB-glutamate and GHB-glycine were identified for the first time. Furthermore, significant changes in concentrations of glycolate and succinylcarnitine were observed. More studies on actual concentrations, detection windows and stability will be necessary to assess the suitability of these markers for forensic routine applications. Untargeted metabolomics proved as a suitable tool for identification of new markers without prior knowledge on possible metabolic pathways. The identification of new GHB markers/metabolites might represent an important step to improve differentiation of endogenous from exogenous GHB and to prolong its detection window in cases of drug facilitated crimes.

Acknowledgements

The authors would like to express their gratitude to Emma Louise Kessler, MD for her generous legacy she donated to the Institute of Forensic Medicine at the University of Zurich, Switzerland for research purposes.

References

[1] S.P. Bessman, W.N. Fishbein. Gamma-Hydroxybutyrate, a Normal Brain Metabolite. *Nature*. **1963**, *200*, 1207.

[2] O.C. Snead, 3rd, B.J. Morley. Ontogeny of gamma-hydroxybutyric acid. I. Regional concentration in developing rat, monkey and human brain. *Brain Res*. **1981**, *227*, 579.

[3] R. Boscolo-Berto, G. Viel, S. Montagnese, D.I. Raduazzo, S.D. Ferrara, Y. Dauvilliers. Narcolepsy and effectiveness of gamma-hydroxybutyrate (GHB): a systematic review and meta-analysis of randomized controlled trials. *Sleep medicine reviews*. **2012**, *16*, 431.

[4] H. Andresen, B.E. Aydin, A. Mueller, S. Iwersen-Bergmann. An overview of gamma-hydroxybutyric acid: pharmacodynamics, pharmacokinetics, toxic effects, addiction, analytical methods, and interpretation of results. *Drug Test Anal*. **2011**, *3*, 560.

[5] J.A. Hall, C.B. Moore. Drug facilitated sexual assault--a review. *Journal of forensic and legal medicine*. **2008**, *15*, 291.

[6] E.E. Kaufman, T. Nelson, D. Miller, N. Stadlan. Oxidation of gamma-hydroxybutyrate to succinic semialdehyde by a mitochondrial pyridine nucleotide-independent enzyme. *J Neurochem*. **1988**, *51*, 1079.

[7] G.F. Zhang, S. Sadhukhan, R.A. Ibarra, S.M. Lauden, C.Y. Chuang, S. Sushailo, P. Chatterjee, V.E. Anderson, G.P. Tochtrop, H. Brunengraber. Metabolism of gamma-hydroxybutyrate in perfused rat livers. *Biochem J*. **2012**, *444*, 333.

[8] S. Sadhukhan, G.F. Zhang, G.P. Tochtrop. Modular isotopomer synthesis of gamma-hydroxybutyric acid for a quantitative analysis of metabolic fates. *ACS Chem Biol*. **2014**, *9*, 1706.

[9] S. Hanisch, N. Stachel, G. Skopp. A potential new metabolite of gamma-hydroxybutyrate: sulfonated gamma-hydroxybutyric acid. *Int J Legal Med*. **2016**, *130*, 411.

[10] I.N. Petersen, C. Tortzen, J.L. Kristensen, D.S. Pedersen, T. Breindahl. Identification of a new metabolite of GHB: gamma-hydroxybutyric acid glucuronide. *J Anal Toxicol*. **2013**, *37*, 291.

[11] R. Brenneisen, M.A. Elsohly, T.P. Murphy, J. Passarelli, S. Russmann, S.J. Salamone, D.E. Watson. Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. *J Anal Toxicol*. **2004**, *28*, 625.

[12] S. Abanades, M. Farre, M. Segura, S. Pichini, A. Pastor, R. Pacifici, M. Pellegrini, R. de la Torre. Disposition of gamma-hydroxybutyric acid in conventional and nonconventional biologic fluids after single drug administration: issues in methodology and drug monitoring. *Ther Drug Monit*. **2007**, *29*, 64.

[13] L.-M. Mehling, T. Piper, A. Spottke, A. Heidbreder, P. Young, B. Madea, M. Thevis, C. Hess. GHB-O-β-glucuronide in blood and urine is not a suitable tool for the extension of the detection window after GHB intake. *Forensic Toxicol*. **2017**, *35*, 263.

[14] T. Piper, L.M. Mehling, A. Spottke, A. Heidbreder, P. Young, B. Madea, C. Hess, W. Schanzer, M. Thevis. Potential of GHB phase-II-metabolites to complement current approaches in GHB post administration detection. *Forensic Sci Int*. **2017**, *279*, 157.

[15] L.M. Mehling, A. Spottke, A. Heidbreder, P. Young, B. Madea, C. Hess, C. Courts. Alterations in gene expression after gamma-hydroxybutyric acid intake-A pilot study. *Int J Legal Med*. **2017**, *131*, 1261.

[16] R.J. Dinis-Oliveira, F. Carvalho, J.A. Duarte, F. Remiao, A. Marques, A. Santos, T. Magalhaes. Collection of biological samples in forensic toxicology. *Toxicology mechanisms and methods*. **2010**, *20*, 363.

[17] F.A. Nasrallah, A.D. Maher, J.R. Hanrahan, V.J. Balcar, C.D. Rae. gamma-Hydroxybutyrate and the GABAergic footprint: a metabolomic approach to unpicking the actions of GHB. *J Neurochem*. **2010**, *115*, 58.

[18] G. Luca, J. Vienne, A. Vaucher, S. Jimenez, M. Tafti. Central and peripheral metabolic changes induced by gamma-hydroxybutyrate. *Sleep*. **2015**, *38*, 305.

[19] M. Palomino-Schatzlein, Y. Wang, A.D. Brailsford, T. Parella, D.A. Cowan, C. Legido-Quigley, M. Perez-Trujillo. Direct Monitoring of Exogenous gamma-Hydroxybutyric Acid in Body Fluids by NMR Spectroscopy. *Anal Chem*. **2017**, *89*, 8343.

[20] M.I. Boxler, T.D. Schneider, T. Kraemer, A.E. Steuer. Analytical considerations for (un)-targeted metabolomic studies with special focus on forensic applications. *Drug Test Anal*. **2018**.
[21] J. Chong, O. Soufan, C. Li, I. Caraus, S. Li, G. Bourque, D.S. Wishart, J. Xia. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res*. **2018**, *46*, W486.
[22] D.S. Wishart, Y.D. Feunang, A. Marcu, A.C. Guo, K. Liang, R. Vazquez-Fresno, T. Sajed, D. Johnson, C. Li, N. Karu, Z. Sayeeda, E. Lo, N. Assempour, M. Berjanskii, S. Singhal, D. Arndt, Y. Liang, H. Badran, J. Grant, A. Serra-Cayuela, Y. Liu, R. Mandal, V. Neveu, A. Pon, C. Knox, M. Wilson, C. Manach, A. Scalbert. HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res*. **2018**, *46*, D608.
[23] L.W. Sumner, A. Amberg, D. Barrett, M.H. Beale, R. Beger, C.A. Daykin, T.W. Fan, O. Fiehn, R. Goodacre, J.L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A.N. Lane, J.C. Lindon, P. Marriott, A.W. Nicholls, M.D. Reily, J.J. Thaden, M.R. Viant. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics*. **2007**, *3*, 211.
[24] T. Barri, L.O. Dragsted. UPLC-ESI-QTOF/MS and multivariate data analysis for blood plasma and serum metabolomics: effect of experimental artefacts and anticoagulant. *Anal Chim Acta*. **2013**, *768*, 118.
[25] Y. Yang, C. Cruickshank, M. Armstrong, S. Mahaffey, R. Reisdorph, N. Reisdorph. New sample preparation approach for mass spectrometry-based profiling of plasma results in improved coverage of metabolome. *J Chromatogr A*. **2013**, *1300*, 217.
[26] A.E. Steuer, K. Arnold, D. Kamber, T. Kraemer. Suitability evaluation of new endogenous biomarkers for the identification of nitrite-based urine adulteration in mass spectrometry methods. *Drug Test Anal*. **2018**.
[27] A.E. Steuer, K. Arnold, T.D. Schneider, M. Poetzsch, T. Kraemer. A new metabolomics-based strategy for identification of endogenous markers of urine adulteration attempts exemplified for potassium nitrite. *Anal Bioanal Chem*. **2017**, *409*, 6235.
[28] M.I. Boxler, G.L. Streun, M.E. Liechti, Y. Schmid, T. Kraemer, A.E. Steuer. Human Metabolome Changes after a Single Dose of 3,4-Methylenedioxymethamphetamine (MDMA) with Special Focus on Steroid Metabolism and Inflammation Processes. *Journal of proteome research*. **2018**, *17*, 2900.
[29] R. Tittarelli, S. Pichini, D.S. Pedersen, R. Pacifici, M. Moresco, F. Pizza, F.P. Busardo, G. Plazzi. Ultra-high-performance liquid chromatography tandem mass spectrometry determination of GHB, GHB-glucuronide in plasma and cerebrospinal fluid of narcoleptic patients under sodium oxybate treatment. *Forensic Sci Int*. **2017**, *274*, 70.
[30] J. Xia, D.S. Wishart. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Current protocols in bioinformatics*. **2016**, *55*, 14 10 1.
[31] J. Mo, L.Y. Lim, Z.R. Zhang. L-Carnitine ester of prednisolone: pharmacokinetic and pharmacodynamic evaluation of a type I prodrug. *Int J Pharm*. **2014**, *475*, 123.
[32] J. Bremer. Carnitine--metabolism and functions. *Physiol Rev*. **1983**, *63*, 1420.
[33] R.R. Ramsay. The carnitine acyltransferases: modulators of acyl-CoA-dependent reactions. *Biochem Soc Trans*. **2000**, *28*, 182.
[34] G.B. Quistad, L.E. Staiger, D.A. Schooley. The role of carnitine in the conjugation of acidic xenobiotics. *Drug Metab Dispos*. **1986**, *14*, 521.
[35] D.S. Millington, T.P. Bohan, C.R. Roe, A.L. Yergey, D.J. Liberato. Valproylcarnitine: a novel drug metabolite identified by fast atom bombardment and thermospray liquid chromatography-mass spectrometry. *Clin Chim Acta*. **1985**, *145*, 69.
[36] A. Zuniga, L. Li. Ultra-high performance liquid chromatography tandem mass spectrometry for comprehensive analysis of urinary acylcarnitines. *Anal Chim Acta*. **2011**, *689*, 77.
[37] G.B. Steventon. *The Amino Acid Conjugations***2002**.
[38] C.P. Badenhorst, E. Erasmus, R. van der Sluis, C. Nortje, A.A. van Dijk. A new perspective on the importance of glycine conjugation in the metabolism of aromatic acids. *Drug Metab Rev*. **2014**, *46*, 343.

[39] T. Kanazu, T. Yamaguchi. Substrate specificity for carnitine and glycine conjugation of branched side-chain and cyclic side-chain carboxylic acids in various experimental animals. *Xenobiotica*. **2009**, *39*, 1.

Table 1: Identified features significantly changed by GHB ingestion.

	Analytical	Feature name	Measured *m/z*	RT (min)	Anova (p)	VIP Score	Fold change	Formula	(Tentative) identification	Identification level [23]
Compound 01	HILIC ESI$^-$	7.18_232.0830m/z	232.0830	7.2	1.8E^{-14}	2.0	Infinity	$C_9H_{15}NO_6$	GHB-glutamate	3
Compound 02	HILIC ESI$^-$	5.35_250.9870m/z	250.9870	5.4	8.1E^{-03}	1.1	Infinity			
Compound 03	HSST ESI$^+$	4.00_259.0789m/z	259.0789	4.0	1.4E^{-15}	2.7	Infinity			
Compound 04	HSST ESI$^-$	3.99_235.0819m/z	235.0819	4.0	3.7E^{-12}	1.9	900.8			
Compound 05	HILIC ESI$^-$	5.70_160.0620m/z	160.0620	5.7	1.7E^{-09}	1.7	765.1	$C_6H_{11}NO_4$	GHB-glycine	3
Compound 06	HSST ESI$^-$	4.58_402.1151m/z	402.1151	4.6	3.3E^{-08}	1.7	219.4			
Compound 07	HILIC ESI$^-$	2.08_104.0472n	103.0399	2.1	5.7E^{-05}	1.5	214.0	$C_4H_8O_3$	GHB	1
Compound 08	HSST ESI$^+$ HILIC ESI$^+$	2.35_248.1496m/z 7.27_248.1487m/z	248.1496 248.1487	2.3 7.3	2.1E^{-13} 2.6E^{-14}	2.7 2.8	91.8 122.4	$C_{11}H_{21}NO_5$	GHB-Carnitine	3
Compound 09	HILIC ESI$^-$	7.38_301.0034m/z	301.0034	7.4	1.1E^{-06}	1.3	73.4			
Compound 10	HILIC ESI$^-$ HILIC ESI$^-$	7.34_205.0624m/z 7.35_249.0526m/z	205.0624 249.0526	7.3 7.3	1.6E^{-04} 4.0E^{-05}	1.2 1.8	39.1 13.6			
Compound 11	HSST ESI$^-$	1.72_159.0295m/z	159.0295	1.7	4.2E^{-10}	1.6	28.0	$C_6H_8O_5$		
Compound 12	HILIC ESI$^-$	4.63_210.0442m/z	210.0442	4.6	4.7E^{-09}	2.2	22.5	$C_6H_{13}NO_5S$		
Compound 13	HILIC ESI$^-$	5.49_192.9821m/z	192.9821	5.5	1.3E^{-04}	1.2	18.3			
Compound 14	HSST ESI$^-$ HSST ESI$^+$	4.57_340.1262n 4.58_340.1233n	385.1252 363.1161	4.6 4.6	4.8E^{-06} 4.8E^{-12}	1.4 2.7	13.3 14.3			
Compound 15	HSST ESI$^+$	5.65_366.2017m/z	366.2017	5.6	3.8E^{-07}	1.6	9.7			

Compound 16	HILIC ESI$^-$	5.49_252.9485m/z	252.9485	5.5	2.3E^{-04}	1.1	9.3			
Compound 17	HSST ESI$^-$	12.12_349.2016m/z	349.2016	12.1	8.8E-03	1.0	6.5			
Compound 18	HSST ESI$^-$ HILIC ESI$^-$	4.17_267.1350m/z 4.92_267.1357m/z	267.1350 267.1357	4.2 4.9	9.3E^{-11} 3.8E^{-09}	2.0 2.1	4.3 6.0	$C_{13}H_{20}N_2O_4$ $C_{13}H_{20}N_2O_4$		
Compound 19	HSST ESI$^-$	0.92_75.0084m/z	75.0084	0.9	1.5E^{-08}	1.7	51.5	$C_2H_4O_3$	Glycolate	1
Compound 20	HSST ESI$^+$	2.94_288.1434m/z	288.1434	2.9	7.1E^{-05}	1.8	5.2	$C_{13}H_{21}NO_6$		
Compound 21	HILIC ESI$^-$	5.44_375.0064m/z	375.0064	5.4	8.9E^{-06}	1.5	3.9			
Compound 22	HILIC ESI$^-$	7.37_307.0561m/z	307.0561	7.4	6.9E^{-04}	1.5	3.5			
Compound 23	HSST ESI$^+$	4.18_268.1419n	269.1497	4.2	1.2E^{-08}	2.3	3.4	$C_{13}H_{20}N_2O_4$		
Compound 24	HILIC ESI$^-$	4.07_342.0328m/z	342.0328	4.1	1.6E^{-03}	1.4	3.4			
Compound 25	HILIC ESI$^+$	3.74_306.0979m/z	306.0979	3.7	1.1E^{-02}	1.2	3.1			
Compound 26	HILIC ESI$^-$	4.63_129.0194m/z	129.0194	4.6	9.9E^{-06}	1.7	2.8	$C_5H_6O_4$		
Compound 27	HILIC ESI$^+$	1.52_127.0384m/z	127.0384	1.5	9.0E^{-03}	1.2	2.6			
Compound 28	HILIC ESI$^+$	0.74_627.2342m/z	627.2342	0.7	2.8E^{-02}	1.1	2.3			
Compound 29	HILIC ESI$^-$	7.05_184.0375n	413.0914	7.0	2.8E^{-05}	1.7	2.2			
Compound 30	HILIC ESI$^-$	5.56_205.0956n	226.0737	5.6	2.3E^{-04}	1.3	2.2			
Compound 31	HILIC ESI$^-$	6.67_228.1449n	265.0935	6.7	1.1E^{-04}	1.5	2.2			
Compound 32	HILIC ESI$^-$	4.94_182.0133m/z	182.0133	4.9	8.5E^{-06}	1.5	2.0			
Compound 33	HILIC ESI$^+$	8.62_271.1025m/z	271.1025	8.6	2.9E^{-03}	1.1	1.9	$C_{10}H_{14}N_4O_5$	Histidinyl-Aspartate	2
Compound 34	HILIC ESI$^+$	7.34_118.0598m/z	118.0598	7.3	2.0E^{-04}	1.3	1.6	$C_3H_7N_3O_2$	Glycocyamine	2

Compound 35	HILIC ESI$^+$	4.48_321.0796m/z	321.0796	4.5	4.5E^{-02}	1.0	1.5			
Compound 36	HILIC ESI$^+$	3.98_264.1103n	297.1440	4.0	3.2E^{-02}	1.1	1.5			
Compound 37	HSST ESI$^-$	1.07_119.0346m/z	119.0346	1.1	6.7E^{-06}	1.5	1.4			
Compound 38	HILIC ESI$^+$	8.61_262.1283m/z	262.1283	8.6	3.2E-^{04}	1.5	1.4	$C_{10}H_{14}N_4O_5$	Succinylcarnitine	2
Compound 39	HILIC ESI$^+$	9.57_225.1218m/z	225.1218	9.6	3.1E^{-02}	1.0	1.4			
Compound 40	HILIC ESI$^+$	7.33_129.0415n	130.0487	7.3	1.6E^{-04}	1.3	1.3	$C_5H_7NO_3$		
Compound 41	HILIC ESI$^+$	7.34_367.1413m/z	367.1413	7.3	4.3E^{-03}	1.1	0.7			
Compound 42	HILIC ESI$^+$	7.69_169.1328m/z	169.1328	7.7	6.6E^{-06}	1.3	0.6			

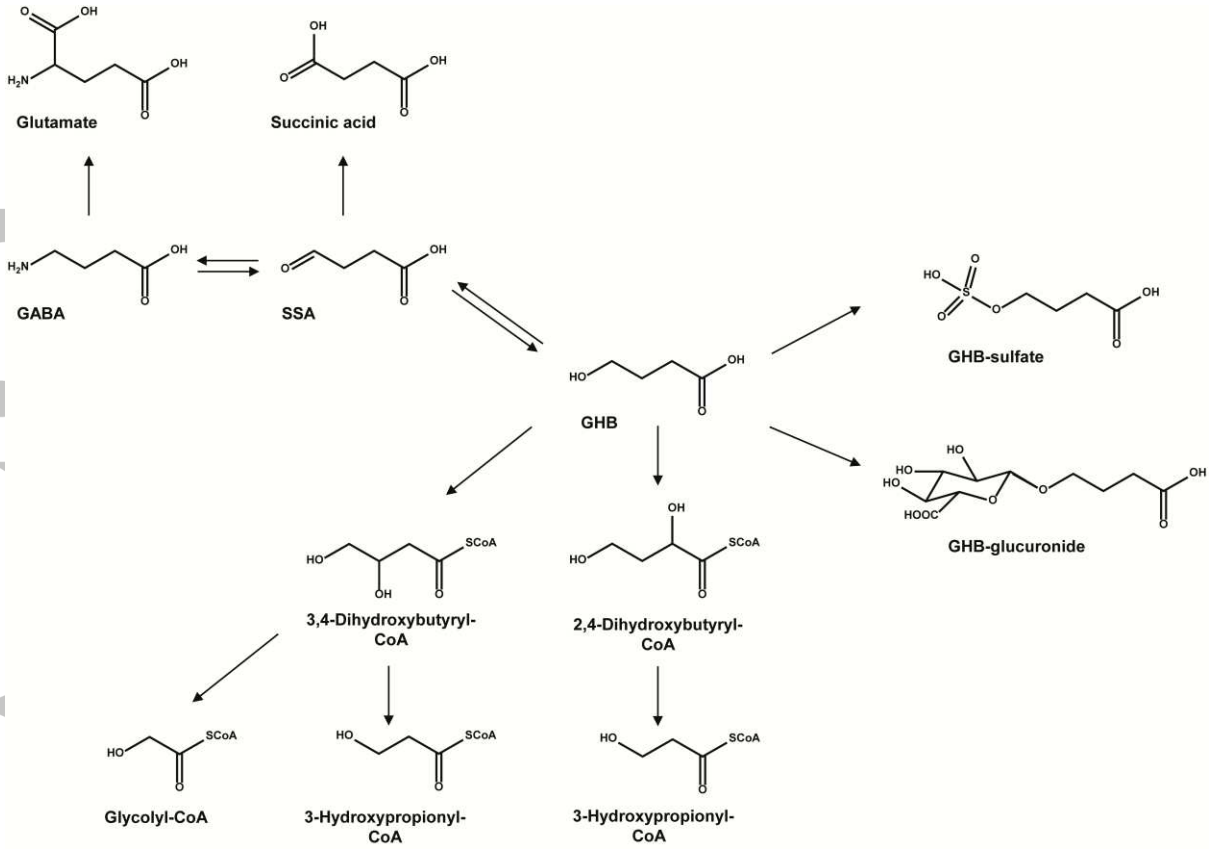

Fig. 1: Metabolism of GHB via different metabolic pathways: oxidation to succinic semialdehyde (SSA), α- and β-oxidation to 2,4-dihydroxybutyryl-CoA and 3,4-dihydroxybutyryl-CoA, respectively and phase II conjugation to GHB-glucuronide and GHB-sulfate.

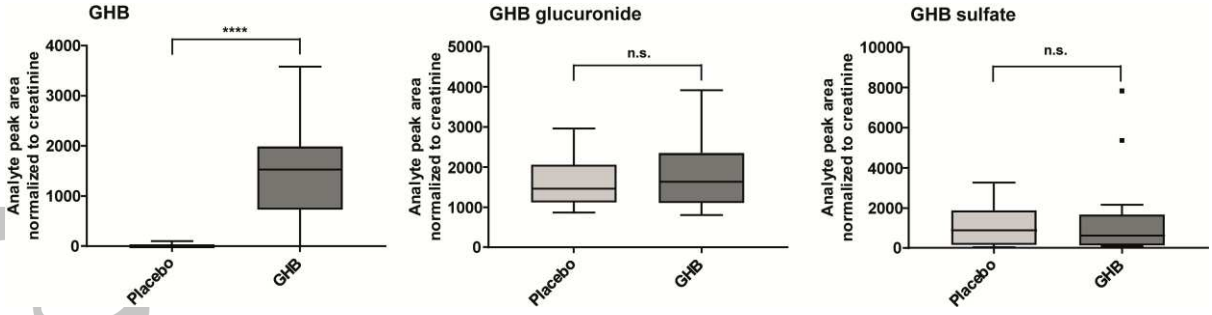

Fig. 2: Blox plots for GHB, GHB glucuronide and GHB sulfate. Depicted are analyte peak area to creatinine ratios for placebo and GHB group (n=19 each). Statistical comparison was performed using a paired t-test ($p < 0.05$): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

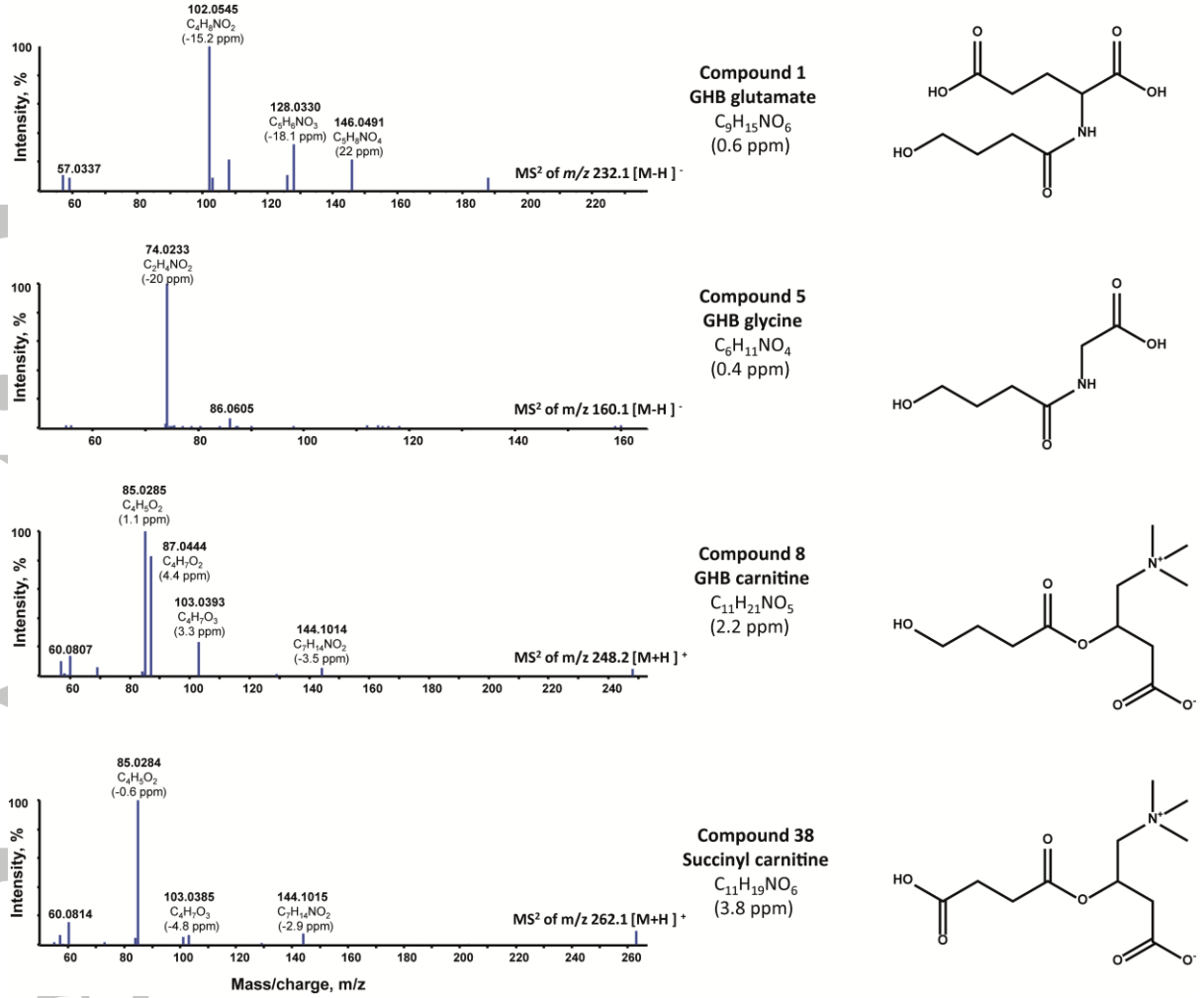

Fig. 3: QTOF MS/MS spectra (collision energy 35 eV, collision energy spread +/- 15 eV) used for identification of significantly changed features. Given are accurate fragment masses of the respective protonated or deprotonated molecular ions, the calculated sum formula of each fragment and corresponding ppm deviation.

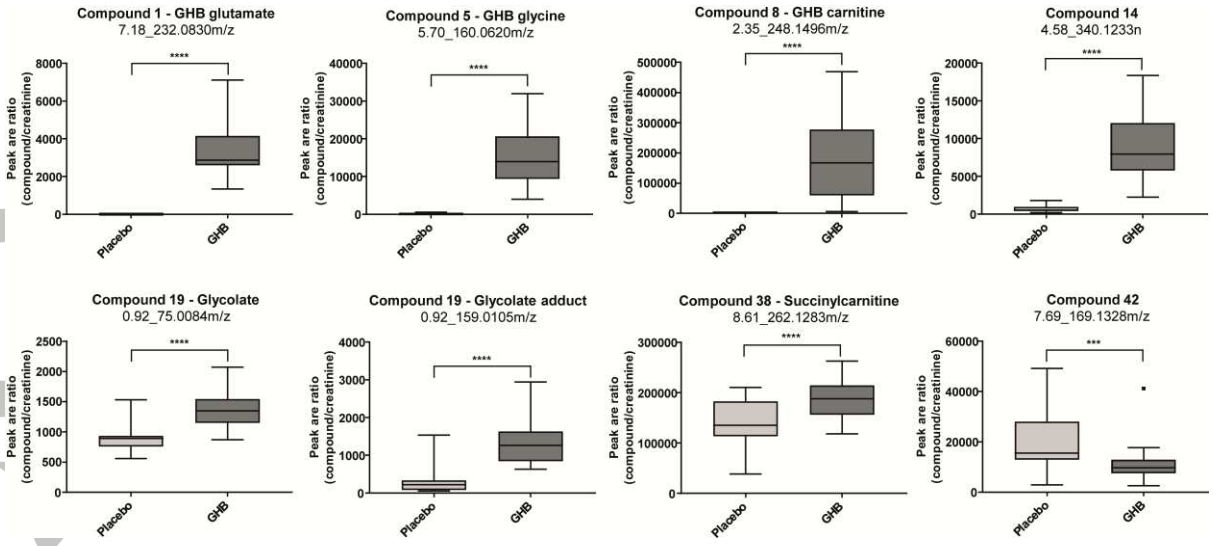

Fig. 4: Blox plots for selected compounds representing typically observed changes between placebo and GHB intake. Depicted are analyte peak area to creatinine peak area ratios for placebo and GHB group (n=19 each). Statistical comparison was performed using a paired t-test ($p < 0.05$): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

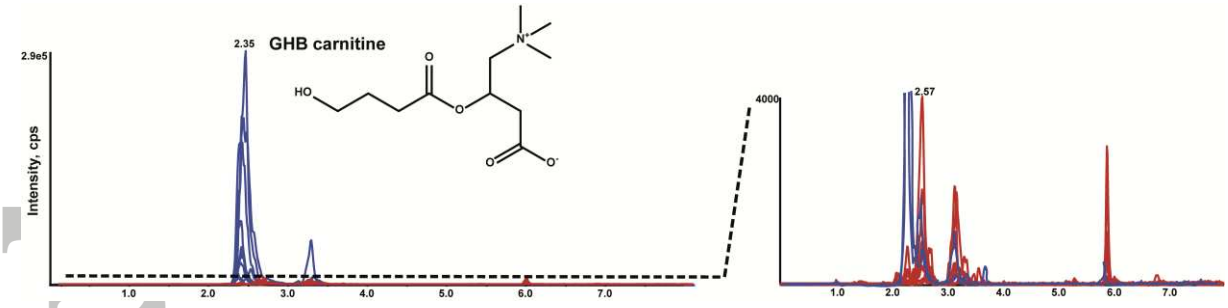

Fig. 5: Extracted ion chromatograms for GHB-carnitine (*m/z* 248. 1492 +/- 10 ppm) of control samples (red; negative for GHB tested by immunoassay, n = 20) and GHB positive authentic samples (blue; tested positive by immunoassay and GC-MS, n = 10); right part: enlargement to illustrate the huge differences between control and authentic samples